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Features of MotA proton channel structure revealed by tryptophan-scanning mutagenesis

(ion channels/membrane proteins)

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ABSTRACT **The MotA protein of *Escherichia coli* is a component of the flagellar motors that functions in transmembrane proton conduction. Here, we report several features of MotA structure revealed by use of a mutagenesis-based approach. Single tryptophan residues were introduced at many positions within the four hydrophobic segments of MotA, and the effects on function were measured. Function was disrupted according to a periodic pattern that implies that the membrane-spanning segments are α-helices and that identifies the lipid-facing parts of each helix. The results support a hypothesis for MotA structure and mechanism in which water molecules form most of the proton-conducting pathway. The success of this approach in studying MotA suggests that it could be useful in structure–function studies of other integral membrane proteins.**

Many bacteria swim by using flagella, thin helical filaments driven at the base by a rotary motor located in the cell membrane (reviewed in ref. 1). The energy for rotation comes from the transmembrane gradient of ions—protons in some species (2) and sodium ions in others (3). Given the observed torque of the motor in *Escherichia coli*, it can be shown that several hundred protons must traverse the membrane to power each revolution (4). The MotA protein is a component of the flagella that functions in transmembrane proton conduction (5), probably acting in concert with another protein called MotB (6–9). Both MotA and MotB are integral membrane proteins (10–13), and both are components in several independently functioning torque generators in the motor (4, 14).

Blair and Berg (5, 11) used random mutagenesis to examine structure–function relationships in the MotA protein. Of 26 different missense mutations that abolished MotA function, all but 2 were found within or adjacent to four hydrophobic segments of the protein. Those results support the suggestion that MotA functions as a transmembrane channel and establish that the segments located in the membrane are most important for function. The mutations had varied effects on side-chain size and polarity and could therefore have disrupted function in several different ways. Because of this chemical variability, the positions of the mutations within the hydrophobic segments did not provide detailed insight into the structure or arrangement of the membrane-embedded segments.

Information on the structure and arrangement of membrane-spanning protein segments has previously been obtained by intensive random mutagenesis targeted to hydrophobic segments (15, 16) or by "cysteine-scanning" mutagenesis, in which single cysteine residues are substituted in many consecutive positions within a segment (17–19). To obtain more detailed information on the structure and arrangement of the membrane-embedded parts of MotA, we have undertaken a systematic mutagenesis of its four membrane-spanning segments. Tryptophan residues, chosen for their large size and moderately hydrophobic character, were introduced at many consecutive positions in each of the hydrophobic segments of the protein and the effects on function were measured. If it is postulated that a large, hydrophobic amino acid such as tryptophan will be tolerated at positions facing the lipid, but most often not at positions inside the protein, the mutational effects can be interpreted in terms of the structure. The mutations disrupted function in a periodic pattern that implies that the four hydrophobic segments of MotA are α-helices and that identifies the parts of each helix directed toward the lipid. The helix faces that could form the channel lining contain very few hydrogen-bonding residues, suggesting that the MotA channel contains water to facilitate the passage of protons.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Materials. Strain MS5037, a gift of M. I. Simon (California Institute of Technology, Pasadena), carries a nonreverting mutation in *motA* that abolishes function. (The defect has not been sequenced.) Strain RP437, a gift of J. S. Parkinson (University of Utah), is wild type for motility and chemotaxis. Site-directed mutagenesis of *motA* used pLS5, a *motA*-containing derivative of phagemid pAlter-1, and the altered sites procedure (Promega). The source of the *motA* gene was pLW3 (20), a gift of R. Macnab (Yale University, New Haven, CT). Plasmid DNA was prepared from single colonies using Qiagen (Chatsworth, CA) cartridges. Mutations were verified by dideoxynucleotide sequencing (21) of double-stranded plasmid DNA. Deoxydenosine 5′-[α-[^{35}S]thio]triphosphate and Sequenase were from Amersham. Deoxyoligonucleotides were synthesized at the University of Utah Protein–DNA Core Facility. Restriction endonucleases were from New England Biolabs.

Motility Assays. The function of mutant MotA proteins was tested by using assays of swarming in soft agar. The *motA* mutations, initially made on pLS5, were subcloned into pLW3 to allow expression at higher levels. Subcloning used either *Hin*dIII and *Mlu* I sites (for mutations in codons 6–185) or *Bgl* II and *Sap* I sites (for mutations in codons 186–215). The mutant derivatives of pLW3 were transformed into strain MS5037, defective in *motA*. Overnight cultures of transformants were grown with shaking at 30°C in tryptone broth (1% tryptone/0.5%NaCl) containing ampicillin (100 μg/ml). One microliter of each saturated culture was spotted onto swarm plates containing tryptone broth, 0.3% agar, and ampicillin (100 μg/ml) and plates were incubated at 30°C. Swarm size was measured at regular intervals and plots of diameter vs. time were fitted to a line. Swarm rates relative to wild-type controls on the same plates are reported.

Tests of Codominance. Cells of the wild-type strain RP437 were transformed with derivatives of pLW3 harboring the *motA* mutations, and swarming rates were assayed by the procedure described above. A control strain harboring wild-type *motA* on pLW3 was included on each plate for comparison.

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Quantification of Mutant MotA Proteins. The mutant MotA variants were expressed from plasmid pLW3, which uses the *trp* promoter, in strain MS5037. Fresh overnight cultures were diluted 1:100 into L broth (typically 10 ml) and cultured at 32°C to an OD_{600} of 0.1. Indoleacrylic acid was added to induce the *trp* promoter (final concentration, 100 μg/ml; added as a 10-mg/ml solution in ethanol), and growth was continued for 14 h. Three milliliters of each culture was harvested by centrifugation, resuspended in spheroplast buffer (0.5 M sucrose/50 mM Tris·HCl, pH 8.0/10 mM EDTA/0.2 mg of lysozyme per ml), and incubated on ice for 1 h. The spheroplasts were sonicated (Branson model 1450; power 3, duty 35%, 1 min) and centrifuged (16,000 × *g*, 20 min, room temperature) to pellet the membranes. Membranes were resuspended in 0.5 ml of water, sonicated, and pelleted as before and resuspended in 30 μl of water. The protein concentration was estimated by the bicinchoninic acid assay (22), and equal amounts of membrane protein ($\approx$10 μg) were loaded onto a SDS/10% polyacrylamide gel and electrophoresed at 15 V/cm for 3 h. The gel was stained with Coomassie brilliant blue and MotA bands were quantified with a video densitometer.

Calculation of Helical Hydrophobic Moments and Variability Moments. Helical hydrophobic moments and variability moments were computed using the expressions in ref. 23, setting the angular rotation parameter ω equal to 100°, corresponding to canonical helical geometry.

RESULTS

Mutagenesis of the MotA Membrane Spanners. The rationale for the tryptophan-mutagenesis experiment is illustrated in Fig. 1. The premise is that the membrane-embedded parts of MotA will consist of a bundle of hydrophobic segments, most likely having regular secondary structure such as α-helices (Fig. 1) or β-sheets. When a bulky residue such as tryptophan is substituted at positions facing the inside of the protein, it should most often disrupt function, while at lipid-facing positions it might be tolerated because the indole side chain can be accommodated in the fluid lipid phase. Thus, tryptophan substitutions should disrupt function according to a pattern that reflects the secondary structure of the membrane spanners and their packing against each other.

MotA has four segments with hydrophobicities in the range expected for membrane spanners (10). Tryptophan residues were substituted, one at a time, in 10–12 consecutive positions in each segment by oligonucleotide-directed mutagenesis of the *motA* gene. The mutations were then transferred onto plasmid pLW3, which expresses MotA at about twice the wild-type level under these conditions (20). The mutant plasmids were transformed into strain MS5037, defective in *motA*, and motility of the resulting strains was tested in a soft-agar swarming assay. The swarming rates measured for 44 tryptophan-substituted MotA variants are shown in Fig. 2 (*Top*).

In four hydrophobic segments, the tryptophan substitutions were tolerated in positions that would be grouped together on one face of an α-helix, as is most readily seen on helical-wheel and helical-net views of the segments (Fig. 3). These results suggest that the hydrophobic segments are α-helices, each with a face that is relatively unconstrained because it faces the lipid. Helices 3 and 4 tolerate tryptophan in fewer positions than helices 1 and 2, suggesting that they are more fully surrounded by other protein segments that cannot accommodate the tryptophan side chain.

Alternative Explanations of the Substitution Effects. Other factors being equal, a substitution should be most disruptive when it causes a large change in side-chain volume. The function of the mutant MotA proteins was not strongly correlated with the volume changes accompanying the tryptophan substitutions, however, implying that the initial residue size did not determine the substitution effects (Fig. 4*A*; correlation coefficient, $R = 0.27$). Similarly, substitutions should be more disruptive when they cause large changes in side-chain hydrophobicity; however, the function of the mutant proteins was also not correlated with the changes in hydrophobicity that accompanied the substitutions (Fig. 4*B*; $R = 0.22$). Thus, the results are best accounted for in terms of side-chain positions rather than size or hydrophobicity.

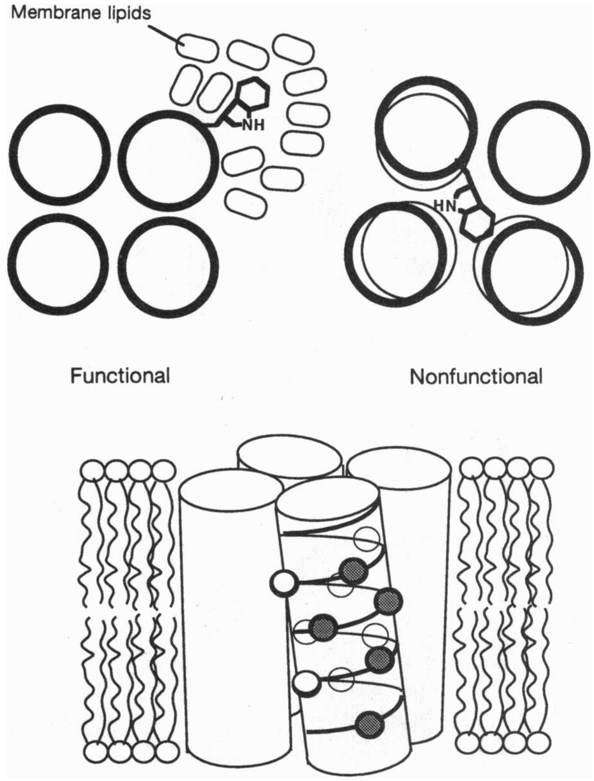

FIG. 1. Strategy for studying secondary structure and packing of the membrane-spanning segments of MotA. The membrane-embedded part of MotA is suggested to consist of a bundle of segments, possibly with regular secondary structure such as the α-helices shown. Schematic cross sections of the channel are shown above, and a side view is shown below. If tryptophan is introduced at several consecutive positions in a membrane spanner, its large side chain should be tolerated at positions facing the lipid, whereas at positions in the protein interior it should usually disrupt function, either by blocking the channel or displacing an adjacent segment. Positions where tryptophan is tolerated (indicated for one segment by shaded circles) should then identify parts of the segments that face outside.

Prediction of Helix Orientations from Hydrophobicities and Sequence Conservation. On average, residues facing the lipid should be more hydrophobic and less well conserved than residues facing the protein interior (23). In favorable cases in which several related protein sequences are known and a pattern in hydrophobicity or residue variability is seen, these properties can be used to identify the face of a membrane spanner most likely to contact the lipid. The most hydrophobic and most variable faces of the MotA helices were determined by using published algorithms (23). The hydrophobic moments of the MotA helices of *E. coli*, and variability moments based on MotA sequences from five species (cf. Fig. 5), are shown on the helical wheels in Fig. 3. The computational approaches had limited success in predicting helix orientations; the hydrophobic moment was in agreement with the tryptophan-substitution effects only for helix 1, and the variability moments did not agree (within 45°) with the substitution effects, or with the computed hydrophobic moments, for any of the helices.

Stability of the Mutant Proteins and Codominance of the *motA* Mutations. The indole side chain of tryptophan could disrupt function directly by blocking the channel or less directly

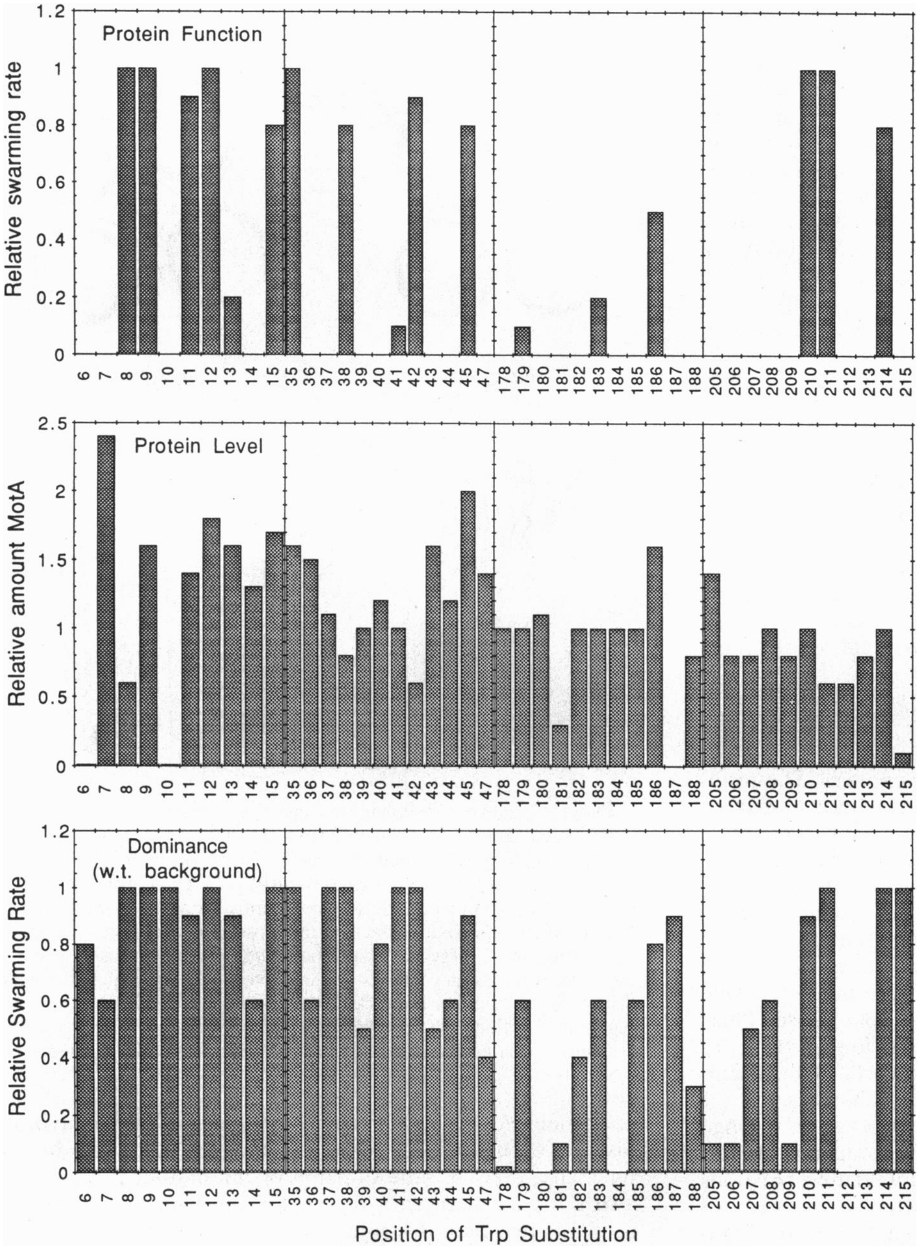

FIG. 2. Effects of tryptophan substitutions in MotA. (*Top*) Function of the tryptophan-substituted MotA variants, assayed by complementation of the *motA*-deficient strain MS5037. The *motA* mutations were on plasmid pLW3. Values are averages of three determinations of swarming rates relative to a wild-type control (strain MS5037 harboring wild-type pLW3). (*Middle*) Relative amounts of the tryptophan-substituted MotA variants in cell membranes. Membranes were isolated from strain MS5037 harboring the mutant variants of *motA* on plasmid pLW3. Values are averages of two determinations, relative to a control strain containing wild-type *motA* on pLW3. (*Bottom*) Codominance of mutant MotA variants. Values are averages of two determinations of swarming rate of the wild-type strain RP437 containing the *motA* mutations on plasmid pLW3 relative to the wild-type strain harboring wild-type *motA* on pLW3.

by contacting adjacent membrane spanners and forcing structural rearrangements that interrupt the conduction pathway. In either case, the positions involved would be important for function, either in forming the channel lining or in making segment contacts. It is also possible that at certain positions tryptophan disrupts function by destabilizing the protein or impeding its insertion into the membrane; positions important for stable folding or membrane insertion might not be identical to those important in the folded protein structure.

To determine whether the mutant proteins were stable, membranes were isolated from cells moderately overexpressing each mutant variant and electrophoresed, and their MotA content was estimated by densitometry. Most of the mutant proteins were present at levels comparable to the wild-type protein (when similarly overexpressed) (Fig. 2 *Middle*). Only three (G6W, V10W, and A187W) were not detected at significant levels. One of these (G6W) is likely to be present at a low level on the basis of other results described below.

As an additional test of whether the mutant proteins were present, their effects on wild-type motility were examined. If a defective MotA protein is present in the membrane and able to make some or all of its normal associations with other motor components, it should impair motility to some degree when expressed in a wild-type strain. Previously, it was found that a large fraction of nonfunctional *motA* mutants were codominant in this assay (5). Exceptions were nonsense mutants in which a sizable part of the protein was not expressed (11). To test their codominance, the mutant variants of pLW3 were transformed into wild-type strain RP437 and swarming rates were measured. Plasmid pLW3 expresses MotA at about twice the wild-type level under these conditions (20). As expected, mutant variants that retained normal function did not interfere with motility when expressed in the wild type. In contrast, most of the nonfunctional tryptophan-substituted mutants (23 of 27) impaired motility significantly (Fig. 2 *Bottom*). Thus, the majority of the mutant proteins are present in the membrane and also able to interact with some other motor component(s). Although not detected in the experiment above, the G6W protein caused a small but reproducible impairment when expressed in the wild type and so must be present, albeit at a low level.

Four mutants, substituted with tryptophan at position 10, 37, 187, or 215, were nonfunctional yet recessive, failing to inhibit

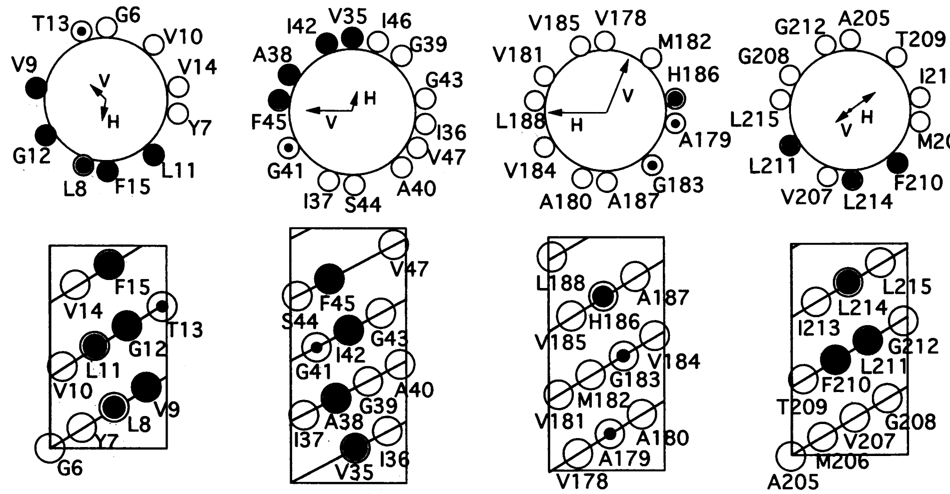

FIG. 3. Results of the tryptophan-scanning mutagenesis experiment, shown on helical-wheel and helical-net projections of the four mutagenized segments of MotA. Position of each amino acid side chain that was altered by mutagenesis is represented by a circle; circles are filled in proportion to the fraction of normal function retained when tryptophan is substituted there. On the helical wheels, the direction and relative magnitudes of the helical hydrophobic moments (H) and sequence variability moments (V) of the segments are also shown. Hydrophobic moments were computed by using the MotA sequence from *E. coli* and the consensus hydrophobicities in ref. 23. Sequence variability moments were computed by using all MotA sequences known (cf. Fig. 5). Magnitudes of the hydrophobic moments and variability moments were as follows: H1, 1.0 and 2.6 (segment length, 23 residues); H2, 0.7 and 6.0 (20 residues); H3, 2.3 and 7.3 (19 residues); H4, 1.2 and 2.5 (23 residues).

motility of the wild type. Two of these proteins (V10W and A187W) were not detected in the membranes and one (L215W) was found at a greatly reduced level (Fig. 2 *Middle*). Interestingly, the fourth recessive variant (I37W) was present at normal levels; this mutant protein might have reduced affinity for its binding partner(s) in the motor.

DISCUSSION

Features of MotA Structure. By systematic substitution of tryptophan residues into many positions in the hydrophobic segments of MotA, we have obtained evidence that these segments are α-helices, each with a face that is relatively unconstrained and thus likely to contact the lipid. Both the sequence of MotA (10) and experiments concerned with its membrane topology (11, 24) suggest that the four segments studied here are the only parts inserted into the membrane. Thus, some or all of these four helices must form most of the proton channel. MotB is also likely to form part of the channel (8); that protein has a single hydrophobic segment (12) and traverses the membrane once (13). Given its length (≈20 residues), it is likely that the MotB segment is also α-helical.

The stoichiometry of subunits in the MotA/MotB channel is not known. Studies of helix-forming model compounds show that a bundle of five helices can form a channel more than large enough to conduct protons (29), so a single copy of each would suffice. Many ion channels have an oligomeric structure, however, including the proton-conducting F_o complex of the ATP synthase, which contains between 10 and 12 copies of a membrane-spanning subunit (30). The MotA/MotB channel is clearly unlike F_o in amino acid sequences and subunit complexity and is likely to have a different architecture.

Implications for Mechanism of Proton Conduction. Whatever the subunit stoichiometry, much of the proton channel appears to be formed from α-helices contributed by MotA. Presumably, the protons follow a relatively polar pathway contained within the protein, not at the interface between protein and lipid. Residues that could form the lining of the proton channel are those facing inside, opposite the face where tryptophan is permitted. Only a few of the residues on these inward-facing surfaces have hydrogen-bonding side chains. These are Y7, T13, Y18, and T21 in helix 1; S44 and E33 in helix 2; and T209 in helix 4. An alignment of the hydrophobic segments of the five known homologs of MotA is shown in Fig.

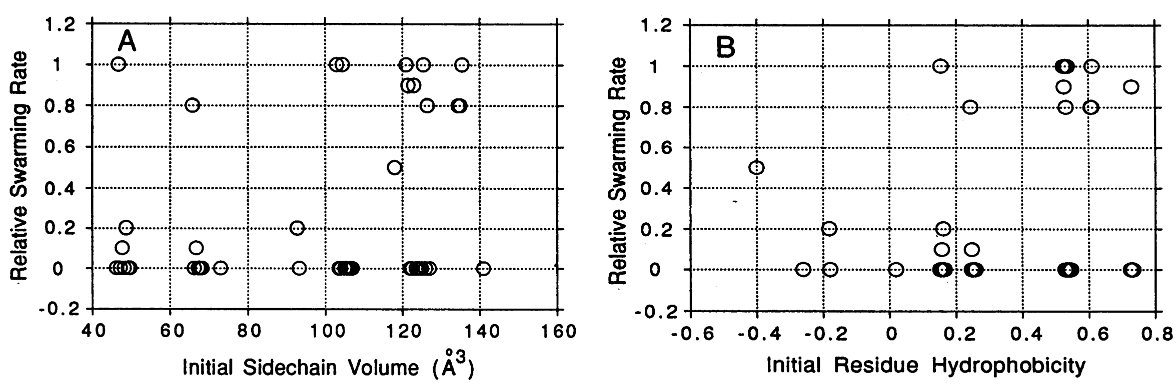

FIG. 4. (*A*) Relative swarming rates of the *motA* tryptophan-substituted mutants, plotted as a function of the side-chain volume of the residue replaced by tryptophan. Side-chain volumes are from ref. 31. In cases in which several data points overlap, they are slightly displaced from each other along the abscissa for clarity. (*B*) Relative swarming rates of the mutants plotted as a function of the hydrophobicity of the residue replaced by tryptophan. Hydrophobicities are from ref. 23. For clarity, some points are slightly displaced along the abscissa as in *A*.

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             Segment 1                   Segment 2
             1                    23     31                 50
E.c.         MLILLGYLVVLGTVFGGYLMTGG      PAELVIIAGAGIGSFIVGNN
V.p.         MQKFLGVLTILVCVFGGYMWAGG      PAEFLIIIGAAAGSLIIGNP
B.s.       MDKTSLIGIILAFVALSVGMVLKGV      PAAILIIIAGTISAVVIAFP
B.m.  MKKIDMLTPIGILIGISMVVFGVISSGG      VPSILIVLGGVFGTLCVSFP
T.p.       MDLASFIGFFGAFAIILMGGILGGS      LPSVFITVGGSYLTLFLAYP

             Segment 3                   Segment 4
            173               191      200                   222
E.c.         PAFGIVAAVMGVVHALGSA       ALIAHAMVGTFLGILLAYGFISP
V.p.         PGFGILAAVGGIIITMQAI       YHVAAALVGTFIGIFGCYCGLDP
B.s.         PTLGVLGAVIGLIAALSHM       HAISAAFVATLLGIFTGYVLWHP
B.m.         PAWGMIGTLVGLVLMLKSL       PDMAIALLTTFYGALLSNLFFQP
T.p.         PGYGML
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FIG. 5. Sequence alignment of hydrophobic segments of MotA from five species. The sequences are from *E. coli* (*E.c.*) (10), *Vibrio parahaemolyticus* (*V.p.*) (25), *Bacillus subtilis* (*B.s.*) (26), *Bacillus megaterium* (*B.m.*) (27), and *Treponema phagedenis* (*T.p.*) (28). Numbers refer to positions in the *E. coli* sequence. Segments mutagenized in the present experiments are underlined; conserved residues are shown in boldface.

5. Among the polar residues in positions that could line the channel, only one (T209) is conserved. When a polar residue in the *E. coli* MotA channel is replaced in another species by a nonpolar residue, there is usually no compensating change elsewhere in the sequence that would restore a polar residue to the vicinity. The single membrane-spanning segment of MotB could also contribute polar residues to the channel, but not very many. Only one polar residue (D32) is conserved in the hydrophobic segments of MotB from four species (12, 25–27). D32 is essential, because a mutation that changed the aspartate to asparagine abolished function completely (7). Tryptophan scanning experiments on MotB show that this residue probably resides in the channel interior (32).

If the proton-conducting pathway in the MotA/MotB channel consisted of a network of hydrogen bonds contributed by amino acid side chains (33), ≈15 hydrogen-bonding groups would be needed, many more than the number available. If the channel were an oligomer with a lining formed from several copies of one or more of the helices, then several polar side chains could face the channel, but they would be clustered at discrete levels and could not form a continuous net of hydrogen bonds traversing the membrane. Because the membrane-spanning segments are α-helices, the backbone amide protons should be relatively immobile and thus unable to contribute to the conduction pathway. We therefore suggest that the MotA/MotB channel contains water molecules to facilitate the passage of protons. This might account for the fact that among the conserved residues that could line the channel, several (G6, G17, A205, and G212) are small.

Tryptophan-Scanning Mutagenesis. Previous scanning mutagenesis studies have used alanine (34) or cysteine (17–19) substitutions. Alanine is used because it is relatively small and nondisruptive; this contrasts with the present experiments in which the aim was to introduce a large, potentially disruptive side chain. Cysteine is also relatively small, but can react with sulhydryl reagents, thereby gaining a large side chain. Cysteine-scanning mutagenesis has been used to probe membrane-spanning segments of the Lac permease (17, 18) and the bacterial chemoreceptor Trg (19). In the case of Trg, cysteine substitutions had effects that, although relatively mild, showed a periodicity consistent with α-helical structure. In membrane spanners of the Lac permease, most cysteine substitutions had minor effects, but when the side-chain bulk was increased by reaction with *N*-ethylmaleimide more severe disruptions were seen, in one case exhibiting helical periodicity (17).

Studies using random mutagenesis suggest that lipid-facing positions can tolerate bulky substitutions. Hinkle *et al.* (16) identified a face of helix VIII in Lac permease that can tolerate diverse, sometimes bulky (e.g., tryptophan, phenylalanine) substitutions. Lemmon *et al.* (15) analyzed a large collection of randomly generated mutants of glycophorin and identified a face on the membrane-spanning helix that is essential for dimer formation. Among the many mutations characterized in that study were several tryptophan substitutions; these had effects consistent with the proposal that tryptophan is tolerated at positions facing the lipid but not at positions contacting the adjacent protein segment in the dimer.

The present results show that lipid-facing regions can in certain cases be distinguished from functionally important interior regions by systematic replacement of native residues by tryptophan. Several features of the MotA structure were revealed in this way; the success of these experiments suggests that valuable insight into the structures of other membrane proteins could be obtained by the same approach. In many instances, information on the secondary structure and orientation of membrane-spanning segments would be useful for understanding mechanisms.

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